United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 409

Supplemental Assay Method for Titrating the Fractions of Combination Avian Encephalomyelitis/Pox Vaccine

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Table of Contents

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1. Introduction

This Supplemental Assay Method (SAM) describes a procedure for titrating a vaccine containing both avian encephalomyelitis (AE) vaccine virus and avian pox vaccine virus. The vaccine is composed of separate preparations of each virus that are mixed together with a suitable stabilizer.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Centrifuge (Beckman J-6B, JS-4.2 rotor)

2.1.2 Humidified, rotating egg incubator (Midwest Incubators, Model No. 252)

2.1.3 Vortex mixer (Thermolyne Maxi Mix II, Model No. M37615)

2.1.4 Microliter pipette (Rainin Pipetman, P1000, or equivalent)

2.1.5 Cool-lite tester (Val-A)

2.1.6 Egg candling light on stand (Speed King)

2.1.7 Etcher electric engraver (Vibro-graver Acme Burgess, Inc.)

2.1.8 Glass 50-ml centrifuge tube, sterile (Kimax)

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 Cotton swabs/cotton balls

2.2.2 Serological pipettes (Falcon)

2.2.3 Pipette tips (Rainin Clean-Pak disposable microliter pipette tips RT-200)

2.2.4 Syringe, 1 cc tuberculin, single use (Becton, Dickinson and Company)

2.2.5 Hypodermic needle, 26-gauge x 3/8-inch (Becton, Dickinson and Company)

2.2.6 Hypodermic needle, 22-gauge x 1 1/2-inch (Becton, Dickinson and Company)

2.2.7 Glass test tubes, 16 x 125-mm with Morton closures

2.2.8 Duco cement

2.2.9 1,1,2-Trichloro 1,2,2-Trifluoroethane (Freon)

2.2.10 Chick embryos from specific-pathogen-free(SPF) source

1. Use 5- to 6-day-old embryos for the AE titration.

2. Use 9- to 11-day-old embryos for the pox titration.

2.2.11 Solutions

1. Tryptose Phosphate Broth (TPB)

TPB	29.5 g
q.s. with distilled or deionized water	1000.0 ml

Sterilize by autoclaving

2. Penicillin/Streptomycin (pen/strep)

penicillin g	500 units/ml
streptomycin	2 mg/ml
q.s. with distilled or deionized water	1000.0 ml

Sterilize by filtration

3. 70% alcohol

ethyl alcohol	70 ml
q.s. with distilled or deionized water	30 ml

4. Iodine, 2% in alcohol

iodine	2 g
ethyl alcohol (70%)	100 ml

2.2.12 Sterile distilled or deionized water

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling and disposal of biological agents, reagents, tissue culture samples, and chemicals. Personnel must also have knowledge of safe operating procedures and policies and Quality Assurance guidelines of the Center for Veterinary Biologics (CVB) or equivalent; and training in the operation of the necessary laboratory equipment listed in **Section 2.1**.

3.2 Preparation of equipment/instrumentation

Operate all equipment/instrumentation according to manufacturers' instructions, and monitor in compliance with current corresponding CVB/National Veterinary Services Laboratories (NVSL) Standard Operating Procedures (SOPs) or equivalent.

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3.3 Preparation of reagents/control procedures

Prepare reference viruses in the same manner as sample preparation.

3.4 Preparation of the sample

3.4.1 AE

Rehydrate 500 doses of vaccine with 10.0 ml sterile purified water. Mix thoroughly. Transfer 1.0 ml of this vaccine to a 50-ml sterile glass centrifuge tube containing 9.0 ml sterile purified water. Mix thoroughly. Add 10.0 ml Freon. Mix on a Vortex mixer for 3 separate 30-second intervals. Centrifuge at 600 X *g* for 10 minutes. The aqueous phase is considered the 10^0 concentration of virus and contains 1 dose in 0.2 ml. Transfer 0.5 ml of the aqueous phase (upper layer/supernate) to a test tube containing 4.5 ml sterile purified water. Make further tenfold dilutions through 10^{-5} using sterile purified water.

3.4.2 Pox

Rehydrate 500 doses of vaccine with 10.0 ml sterile purified water. Mix thoroughly. Transfer 0.5 ml of this vaccine to a sterile test tube containing 4.5 ml of TPB. This is considered the 10^0 concentration and contains 1 dose in 0.2 ml. Make further tenfold dilutions, transferring 0.5 ml vaccine to 4.5 ml TPB, up through 10^{-6}.

4. Performance of the test

4.1 AE

Inoculate dilutions 10^{-1} through 10^{-5} into the yolk sac using 10 embryos per dilution. Inoculate 0.2 ml per embryo. Also have 20 uninoculated negative controls. Incubate the embryos and calculate the titer according to the criteria specified in the Code of Federal Regulations, Title 9 (9 CFR) 113.325(c)(2)(i).

4.2 Pox

Inoculate dilutions 10^{-2} through 10^{-6} onto the dropped chorioallantoic membrane (CAM) using at least 6 embryos per dilution. Inoculate 0.2 ml per embryo. Incubate the embryos and calculate the titer according to the criteria specified in 9 CFR 113.326.

5. Interpretation of the test results

5.1 Controls

Titrate a known positive reference virus with each group of titrations. The titer of the positive reference virus must be within the established range for the test results to be valid.

5.2 Calculating the titer

Determine the log 10 EID_{50} titer using the method of Reed and Muench. This dilution and inoculation procedure allows for a direct readout on a per-dose basis. Round to 1 decimal.

5.3 Retests

Conduct retests as required by 9 CFR 113.8(b) and requirements of minimum release in firm's current Outline of Production, Part V.

5.4 Evaluation of test results

5.4.1 The 9 CFR 113.8(b) defines the criteria for a satisfactory/unsatisfactory serial.

5.4.2 The firm's requirements of minimum release/stability titers for each vaccine are listed in the current Outline of Production, Part V, for the specific product code.

6. Report of test results

Titers are reported out as EID_{50} per bird dose.

7. References

7.1 Code of Federal Regulations, Title 9, U.S. Government Printing Office, Washington, D.C., 2004.

7.2 Reed, L.J., and H. Muench. 1938. A simple method of estimating 50% endpoints. Am. J. Hyg. 27:493-497.

8. Summary of revisions

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- **2.2.9/3.4.1** Chloroform has been changed to 1,1,2-Trichloro 1,2,2-Trifluoroethane (Freon)
- **2.2.11(2)** 1.775 g has been changed to 500 units/ml of penicillin and 100 g has been changed to 2 mg/ml of streptomycin
- **2.2.11** The “Normal Saline” formula has been deleted.